Glycogen Synthase Kinase-3β Is a Functional Modulator of Serotonin-1B Receptors

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ABSTRACT

Glycogen synthase kinase-3 (GSK3) is a constitutively active protein kinase that is involved in neuronal regulation and is a potential pharmacological target of neurological disorders. We found previously that GSK3β selectively interacts with 5-hydroxytryptamine-1B receptors (5-HT1BR) that have important functions in serotonin neurotransmission and behavior. In this study, we provide new information supporting the importance of GSK3β in 5-HT1BR-regulated signaling, physiological function, and behaviors. Using molecular, biochemical, pharmacological, and behavioral approaches, we tested 5-HT1BR’s interaction with Gαo2 and β-arrestin2 and 5-HT1BR-regulated signaling in cells, serotonin release in mouse cerebral cortical slices, and behaviors in wild-type and β-arrestin2 knockout mice. Molecular ablation of GSK3β and GSK3 inhibitors abolished serotonin-induced change of 5-HT1BR coupling to Gαo2 and associated signaling but had no effect on serotonin-induced recruitment of β-arrestin2 to 5-HT1BR. This effect is specific for 5-HT1BR because GSK3 inhibitors did not change the interaction between serotonin 1A receptors and Gαo2. Two GSK3 inhibitors, N-(4-methoxybenzyl)-N’-(5-nitro-1,3-thiazol-2-yl)urea (AR-A014418) and 3-(5-bromo-1-methyl-1H-indol-3-yl)-4-(benzofuran-3-yl)pyrrole-2,5-dione (BIP-135), efficiently abolished the inhibitory effect of the 5-HT1BR agonist anpirontline on serotonin release in mouse cerebral cortical slices. GSK3 inhibitors also facilitated the 5-HT1BR agonist anpirontline-induced behavioral effect in the tail suspension test but spared anpirontline-induced locomotor activity. These results suggest that GSK3β is a functional selective modulator of 5-HT1BR-regulated signaling, and GSK3 inhibitors fine-tune the physiological and behavioral actions of 5-HT1BR. Future studies may elucidate the significant roles of GSK3 in serotonin neurotransmission and implications of GSK3 inhibitors as functional selective modulators of 5-HT1BR.

Introduction

Glycogen synthase kinase-3 (GSK3) (Embi et al., 1980) is a multifunctional protein kinase that phosphorylates and modulates many protein substrates (Doble and Woodgett, 2003). GSK3α and GSK3β are paralogous proteins that share 84%

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ABBREVIATIONS: GSK3, glycogen synthase kinase-3; BRET, bioluminescence resonance energy transfer; FST, forced swim test; GPCR, G protein-coupled receptor; 5-HT1BR, 5-hydroxytryptamine-1B receptor; 5-HT1AR, 5-hydroxytryptamine-1A receptor; 8-OH-DPAT, 8-hydroxy-2-(di-n-propyl)aminotetralin; TST, tail suspension test; AR-A014418, N-(4-methoxybenzyl)-N’-(5-nitro-1,3-thiazol-2-yl)urea; GFP, green fluorescent protein; YFP, yellow fluorescent protein; Ric, Renilla reniformis luciferase; HEK, human embryonic kidney; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; ANOVA, analysis of variance; HA, hemagglutinin; shRNA, short hairpin RNA; SB216641, N-[3-(dimethylamino)ethyl]oxy]-4-methoxyphenyl]-2'-methyl]-4'-(5-methyl-1,2,4-oxadiazol-3-yl)-[1',3'-biphenyl]-4'-carboxamide; SB216763, 3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrole-2,5-dione; SB224289, 1'-methyl-5-[2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)phenyl]-4'-ylcarboxy]-2,3,6,7-tetrahydrospiro-furo[3,2-f]indole-3,4'-piperidine; WAY100635, N-[2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl]N'-2-pyridyldicyclohexane-carboxamide; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]propanesulfonate; BIP-135, 3-(5-bromo-1-methyl-1H-indol-3-yl)-4-(benzofuran-3-yl)pyrrole-2,5-dione.

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Introduction

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Joep, 2010) and neurodegenerative disorders (Muyllaert et al., 2008). However, to better develop disease-specific treatment by normalizing GSK3 activity, it would be important to identify selective GSK3 substrates that have specific neurological functions.

We found previously that GSK3β selectively interacts with serotonin 1B receptors (5-HT1BR) (Chen et al., 2009). 5-HT1BR belong to the Class A G protein-coupled receptors (GPCRs) that activate the inhibitory G protein (Gi) to inhibit adenyl cyclase and cAMP production (Raymond et al., 2001) and to activate trophic factor-regulated signaling, such as Akt (Leone et al., 2000). 5-HT1BR autoreceptors in serotonin neurons are located at axon terminals (Sari, 2004) and function to inhibit serotonin release in serotonergic neuron-innervated brain regions (Riad et al., 2000). 5-HT1B heteroreceptors interact with other neurotransmitter systems, such as GABA, dopamine, glutamate, and acetylcholine (Sari, 2004). Upon a balanced activity between their autoreceptors and heteroreceptors, 5-HT1BR regulate various behaviors such as mood, anxiety, reward, activity, and aggression (Clark and Neumaier, 2001; Miczek et al., 2004; Sari, 2004). Although 5-HT1BR play a substantial role in regulating serotonin neurotransmission and related behaviors, pharmacological treatment targeting 5-HT1BR has not been fully investigated.

The selective interaction between GSK3β and 5-HT1BR makes the molecular mechanism an attractive target for drug development once the physiological significance of this mechanism is fully elucidated. In this study, we examined the roles of GSK3 on 5-HT1BR coupling to G protein β-arrestin2, the two major intracellular protein mediators of GPCR signaling. We also investigated the effect of GSK3 in modulating 5-HT1BR-mediated cAMP and Akt signaling, serotonin release, and behaviors. The results show that active GSK3β selectively modulates G protein-coupled 5-HT1BR signaling, GSK3 inhibitors act to maintain serotonin output by abolishing the negative effect of 5-HT1BR autoreceptors, and GSK3 inhibitors differentially affect 5-HT1BR-mediated behaviors.

**Materials and Methods**

**Chemicals.** Anpirtoline, N-[3-[3-(dimethylamino)ethoxy]-4-methoxyphenyl]-2'-methyl-4'-[5-methyl-1,2,4-oxadiazol-3-yl]-[1H-biphenyl]-4-carboxamide (SB216641), 3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (SB216763), and 1'-methyl-5-[(2'-methyl-4'-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl]-2,3,6,7-tetrahydropyrazolo[2,3-f]indole-3,4'-piperidine (SB224289) (Tocris Bioscience, Ellisville, MO); serotonin, 8-hydroxy-2-(N,N-dipropylaminotetralin (8-OD-PAT), N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N'-2-pyrindinylcyclohexanecarboxamide (WAY100635), and N-(4-methoxybenzyl)-N'-[5-nitro-3-thiazol-2-yl]urea (AR-A014418), Sigma-Aldrich, St. Louis, MO; [H]serotonin (PerkinElmer Life and Analytical Sciences, Waltham, MA); and 3-(5-bromo-1H-indol-3-yl)-4-(benzofuran-3-yl)pyrrole-2,5-dione (BIP-135) and (benzofuranyl-3-yl-indol-3-yl)maleimide compounds M1 and M13 (A. P. Kozikowski, University of Illinois, Chicago, IL) were obtained. Chemicals and drugs are dissolved in distilled water, dimethyl sulfoxide, or ethanol (EtOH) in a concentrated solution before they were used for experiments.

**DNA Constructs and Mutagenesis.** Mouse 5-HT1BR cDNA (Per Svenningsson, Karolinska Institute, Sweden); rat serotonin 1A receptor (5-HT1AR) cDNA (P. Albert, University of Ottawa, Ottawa, ON, Canada); mouse GSK3β, R66A-GSK3β, K58,86A-GSK3β, and S9A-GSK3β (R. S. Joep, University of Alabama at Birmingham, Birmingham, AL); human G protein α and human β-arrestin2 (Missouri S&T cDNA Resource Center, Rolla, MO); peDNA6 vector (Invitrogen, Carlsbad, CA); pEGFP-C1 and pEYPF-C1 (Clontech, Mountain View, CA); pRL-TK vector (Promega, Madison, WI). 5-HT1BR and 5-HT1AR cDNAs were subcloned into pEGFP-C1 vector with N-terminal GFP, or pRL-TK vector with C-terminal luciferase. Human G protein α and β-arrestin2 cDNAs were subcloned into pEYPF-C1 vector with N-terminal YFP for G protein α, and C-terminal YFP for β-arrestin2. Mutagenesis of mouse 5-HT1BR was performed as described previously (Chen et al., 2009). All constructs were verified by DNA sequencing before cellular transfection.

**RNA Interference of GSK3.** Short hairpin RNA (shRNA) sequences for human GSK3β, GSK3α, and nontargeting shRNA (Sigma-Aldrich) are constructed into lentivirus particles. The shRNA-targeting sequences are shown: GSK3β: b1, CCCAATACACAGAATTTA; b2, GACACTAAAGTGATTGGAAAT; and b3, CACCTAGTATACCTCTAGTA; GSK3α: a1, GACTAGGGGCAGAGGTAAT; a2, GAGTGTTAAGTCCCTCAGATT; and a3, CTACTCTGGTTCTCGG-TACTAC; and nontargeting shRNA, CGCCGACAAGATGGAAGGCACCAACTCGAGTTGGTCTCCTATGGTTGTTTT. Although embryonic kidney (HEK) 293 cells were transduced with shRNAs for human GSK3β, GSK3α, or nontargeting shRNA (control). Cell lines expressing shRNA clones were selected by the antibiotic puromycin (1 μg/ml). All RNA interference-induced GSK3 knockdowns were confirmed by immunoblotting using the anti-GSK3α/β antibody.

**Cell Culture and Transfection.** HEK293 cells and Chinese hamster ovary (CHO) cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and penicillin (100 U/ml)/streptomycin (0.1 mg/ml). For transient transfection of DNA, cells were grown to 70% confluence, and DNAs were transfected into cells using the FuGene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) following the manufacturer’s protocol. Stable-transfected cell lines were selected and maintained in media containing corresponding antibiotics. For experiments, cells were placed in serum-free media before treatment with inhibitors and/or activators. All experiments using transiently transfected DNA constructs were performed in HEK293 cells except for Akt phosphorylation and cAMP assays that were performed in CHO cells in which the signaling cascades are well preserved.

**Bioluminescence Resonance Energy Transfer Assay.** HEK cells stably expressing 5-HT1BR-Rluc or 5-HT1AR-Rluc were transfected with YFP, YFP-GSK3β, YFP-G protein α, or β-arrestin2-YFP. Luciferase activity was measured using coelenterazine f or coelenterazine h (Invitrogen) as the substrate (5 μM). YFP expression was confirmed by measuring fluorescence signal acquired by excitation at 485 ± 10 nm and emission at 528 ± 10 nm using the Synergy 2 spectrofluorometer. For real-time bioluminescence resonance energy transfer (BRET), cells were first treated with PBS-EDTA, washed, and suspended with 0.1% glucose in PBS containing 0.5 mM MgCl2. Cells were transferred into white opaque 96-well plate and incubated at 37°C for 30 min before addition of coelenterazine h. Measurement of the dynamic BRET signal was initiated 1 min later. Serotonin was added 21 s after BRET measurement was initiated, and the measurement was continued for 10 min to assure that any change of dynamic protein interaction after treatment was observed for a sufficient length of time. For fixed-time BRET measurement, cells were lifted from culture dishes after they were treated with drugs, washed with PBS, and transferred into white opaque 96-well plate. BRET signal was detected 1 min after addition of coelenterazine f into each well. To measure BRET signals, emission was detected at the wavelength of 485 ± 10 and 528 ± 10 nm, corresponding to the maxima of the emission spectra for Rluc and YFP, respectively. The BRET ratio was calculated using the equation (emission at 528 ± 10 nm) / (emission at 485 ± 10 nm × CF) / (emission at 485 ± 10 nm) (Angers et al., 2000), where CF is (emission at 528 ± 10/emission at 485 ± 10 nm measured in cells transfected with 1 μg of a DNA vector without YFP).
Immunoprecipitation and Immunoblotting. Cells coexpressing wild-type or mutant GFP-5-HT1BR and HA-GSK3β, HA-GSK3α, or HA-β-arrestin2 were lysed by feeding in a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1.5% CHAPS, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml pepstatin, 0.1 μM β-glycerophosphate, 1 mM phenylmethylsulfonfyl fluoride, 1 mM sodium vanadate, and 100 mM 10% polyacrylamide gels and immunoblotted with antibodies against GSK3β (Abcam Inc., Cambridge, MA) and anti-HA (Covance Research Products, Princeton, NJ) antibodies. Protein bands were detected with enhanced chemiluminescence (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

For immunoblotting GSK3 and Akt, protein lysates from CHO cells were prepared in a buffer containing 10 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.5% NP-40, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml pepstatin, 0.1 mM β-glycerophosphate, 1 mM phenylmethylsulfonfyl fluoride, 1 mM sodium vanadate, and 100 mM 10% polyacrylamide gels and immunoblotted with antibodies specific for total GSK3α/β (Millipore, Billerica, MA), phospho-T308-Akt, phospho-S473-Akt, and total Akt (Cell Signaling Technology, Danvers, MA).

cAMP Assay. The level of cAMP was measured in CHO cells using an enzyme immunoassay kit (Direct BioTrak; Amersham/GE Healthcare) (Chen et al., 2009). To minimize the potential nonspecific effects on GSK3 by other inhibitors, all experiments with cAMP measurement in this study were conducted in the presence of phosphodiesterase inhibitors. After pharmacological treatments, cells were lysed in a buffer containing 2.5% dodecylmaltoside ammonium bromide. Duplicate lysates were transferred into individual wells of a 96-well plate precoated with donkey anti-rabbit IgG. After incubation with rabbit cAMP antiserum and cAMP-horseradish peroxidase conjugates, enzyme reaction was started by the addition of peroxidase substrate and hydrogen peroxide. The radioactivity of all fractions was calculated as the following: counts per minute × 100/baseline counts per minute. Data are processed by OriginPro 8.1 (OriginLab Corp., Northampton, MA), and the radioactivity of all fractions was calculated as the following: counts per minute × 100/baseline counts per minute. Data are processed by OriginPro 8.1 (OriginLab Corp., Northampton, MA), and the radioactivity of all fractions was calculated as the following: counts per minute × 100/baseline counts per minute.

Animals. The Institutional Animal Care and Use Committee at the University of Alabama at Birmingham approved animal use in this study. Twelve-week-old male C57BL/6 mice (Fredrick Cancer Research, Fredrick, MD) were housed four to five per cage with free access to food and water in a 12-h light/dark cycle animal facility for 1 week before used for experiments. Male homozygous β-arrestin2 knockout mice in C57BL/6 background (R. J. Lefkowitz, Duke University, Durham, NC) (Bohn et al., 1999) were continuously bred from homozygous breeders and were used along with strain-matched wild-type mice for behavior tests.

Serotonin Release. Male C57BL/6 wild-type mice were sacrificed by decapitation, and brains were rapidly dissected in ice-cold Krebs buffer [121 mM NaCl, 1.2 mM MgCl2, 1.3 mM CaCl2, 25 mM NaHCO3, 1.0 mM NaH2PO4, 10 mM glucose, 13.5 mM KCl, 50 μM l-tyrosine, 10 μM nomifensine maleate (dapamine reuptake inhibitor), 50 μM l-tryptophan, and 10 μM imipramine (serotonin reuptake inhibitor)]. Transverse cerebral cortical slices (300 μm) were prepared by a tissue chopper (McILWAIN, Surrey, England) and rinsed in a cold Krebs buffer that was equilibrated with 95% O2 and 5% CO2. Brain slices were labeled with [3H]serotonin (1.3 μCi/ml) in Krebs buffer with 95% O2 and 5% CO2 at 37°C for 30 min, transferred into the perfusion chambers (10–11 slices/chamber), and superfused with aerated (95% O2 and 5% CO2) and preheated (37°C) Krebs buffer. The flow rate was kept at 0.5 ml/min by a Bio-Rad multichannel peristaltic pump (Econo Pump; Bio-Rad). Beginning at 61 min of perfusion, the superfusion fluid was collected at 2 ml/min. For GSK3 inhibitor treatment, the Krebs buffer was supplemented with AR-A014418 (5 μM), BIP-135 (1 μM), or 0.1% EtOH (vehicle) at 20 min of perfusion and continued throughout. Serotonin release was induced by potassium chloride (KCl, 50 mM) at 63 min (P1) and at 83 min (P2), with the perfusion buffer replaced by a new Krebs buffer between P1 and P2 at 78 min when aniprotine (5 μM) was added. Collection of superfusion fluid continued until 98 min.

At the end of the experiment, the brain slices from each chamber were collected and homogenized in 200 μl of 1 N HCl containing 0.1% Triton X-100. Superfusion fluid from each fraction and tissue homogenate (200 μl) was mixed with scintillation liquid (5 ml of Universal; MP Biomedicals, Solon, OH), and radioactivity was counted by a scintillation spectrometer (Multi-Purpose Scintillation Counter, LS-6500; Beckman Coulter, Fullerton, CA). To calculate [3H]serotonin release, the radioactivity from fractions collected at 61 to 63 min were averaged as a baseline for the first KCl stimulation, and fractions at 81 to 83 min were averaged for the second KCl stimulation. The radioactivity of all fractions was calculated as the following: counts per minute × 100/baseline counts per minute. Data are processed by OriginPro 8.1 (OriginLab Corp., Northampton, MA), and the level of cAMP was calculated against a protein sample. To calculate the area under the curve, the area surrounding the fitting line and baseline. Effect of a drug was determined by the ratio of the second overflow (P2) to the first overflow (P1).

Drug Treatment. For intracerebroventricular infusion of experimental drugs, mice were anesthetized with ketamine and xylazine (100 mg/kg/10 mg/kg) to place a guide cannula stereotactically (posterior 0.8 mm and right 1.6 mm to the bregma; depth, 2.2 mm). Five days later, GSK3 inhibitors AR-A014418 (0.3 nmol), BIP-135 (0.08 nmol) or vehicle (10% dimethyl sulfoxide) was infused into the right ventricle via an internal cannula 90 min before aniprotine (4 mg/kg) or saline injection intraperitoneally at a volume of 5 μl/γ body weight.

Behavior Tests. The tail suspension test (TST) was conducted using an automated testing system (Med Associates Inc., St. Albans, VT) (Polter et al., 2010). Movement is measured for 6 min, and the immobility time was recorded with the Med Associates software and calculated as the time the force of movement was below a preset threshold. Immobility was recorded as each 2-min bin and the last 4 min of testing. For drug treatment, aniprotine or saline was given 30 min before testing. The locomotor activity was tested in a Plexiglas open field (Med Associates), and activity was monitored using the activity monitoring software (Med Associates) (Polter et al., 2010). Mice were allowed to habituate in the open field for 15 min before aniprotine or saline injection, followed by an additional 30-min testing in the open field. Travel distance during each 5-min block and total distance traveled during the 15-min pretreatment and the 30-min post-treatment time periods were recorded.

Data Analysis and Statistics. All experiments and treatments were repeated for statistical analysis using SPSS (SPSS Inc., Chicago, IL). The analysis of variance (ANOVA) with post hoc or Student's t test was applied as appropriate for each set of experiments. Values are expressed as mean ± S.E.M. and are considered significant when p < 0.05.

Results

As a G1-coupled GPCR, 5-HT1BR, communoprecipitated with Gα13 in either the absence or the presence of serotonin (10 μM), with serotonin treatment transiently increased the protein association (Fig. 1A). Because GSK3β mainly interacts with the Ser154/Thr158 consensus GSK3 phosphorylation site located in the second intracellular loop of mouse 5-HT1BR (Chen et al., 2009), we communoprecipitated the...
Fig. 1. Association and dynamic interaction between 5-HT1BR and G\(_{\alpha}\). A, HEK293 cells were transfected with GFP-tagged 5-HT1BR wild-type (1BR-WT), S154A mutant (1BR-S154A), or T158A mutant (1BR-T158A), and cotransfected with HA-Gi\(_{\alpha}\). Some of these cells were treated with serotonin (5-HT, 10 \(\mu\)M) for the indicated time. Whole-cell lysates were immunoprecipitated with anti–HA-conjugated agarose beads. Both immunocomplex (IP: HA) and cell lysate (lysate) were immunoblotted for GFP and HA. Optimal density of immunoblotted GFP-5-HT1BR-WT was calculated as the percentage of baseline (0 min serotonin treatment). Mean \(\pm\) S.E.M., \(n = 3\) to 5 at each time interval; *, \(p < 0.05\) in ANOVA when treatment was compared with baseline (no serotonin). Cells cotransfected with a GFP vector (no 5-HT1BR) and HA-Gi\(_{\alpha}\) were also immunoprecipitated with anti-HA as negative control. B, wild-type 5-HT1BR-Rluc-expressing HEK293 cells were transfected with YFP or YFP-Gi\(_{\alpha}\) (1 \(g\)), and real-time BRET was measured in the absence or the presence of serotonin (10 \(\mu\)M).
S154A and T158A mutant 5-HT1BR with G\textsubscript{\alpha}2. Both mutant receptors associated with G\textsubscript{\alpha}2, but in contrast to wild-type receptors, serotonin did not change the association between the mutant 5-HT1BR and G\textsubscript{\alpha}2 (Fig. 1A), suggesting that only serotonin-induced, but not the resting state of 5-HT1BR-G\textsubscript{\alpha}2 association is affected by GSK3.

To further determine the effect of GSK3 on the dynamic interaction between 5-HT1BR and G\textsubscript{\alpha}2, we used the BRET assay that measures the proximity of two proteins in living cells (Angers et al., 2000). In intact cells, wild-type 5-HT1BR-Rluc and YFP-G\textsubscript{\alpha}2 interacted with each other at the resting state with a small but reliably detectable oscillation (Fig. 1B). The oscillatory interaction was not detected between 5-HT1BR-Rluc and YFP. Treatment with serotonin (10 \( \mu \text{M} \)) transiently stabilized the oscillation at the maximum level of interaction for approximately 100 s before it returned to oscillation. In the absence of serotonin, both the S154A and the T158A mutant 5-HT1BR also interacted with G\textsubscript{\alpha}2 in an oscillatory pattern, but different from the wild-type 5-HT1BR, serotonin did not cause a transient stabilization of the interaction (Fig. 1C).

We also tested the interaction between 5-HT1BR and G\textsubscript{\alpha}2 after termination of serotonin treatment. In this experiment, cells were preincubated with serotonin for 15 min and then were washed to remove serotonin, followed by measuring the 5-HT1BR-G\textsubscript{\alpha}2 interaction using the fixed-time BRET. An YFP fluorescent intensity-dependent interaction between 5-HT1BR and G\textsubscript{\alpha}2 (maximum at the fluorescent intensity of 12.8 \times 10\(^{4}\) with 1 \( \mu \text{g} \) of YFP-G\textsubscript{\alpha}2 DNA) was reduced significantly (Fig. 1D). This was not a resting state interaction, because it required pre-serotonin treatment and was concentration-dependently blocked by the 5-HT1BR antagonist SB224289 and SB216641 applied before serotonin treatment (Fig. 1E). Different from the wild-type 5-HT1BR, serotonin pretreatment did not cause a reduction in interaction between the mutant S154A or T158A mutant 5-HT1BR and G\textsubscript{\alpha}2 (Fig. 1F). These results indicate that disruption of GSK3\(\beta\) binding to the intracellular loop-2 of 5-HT1BR (Chen et al., 2009) drastically alters the activity-dependent conformational change between 5-HT1BR and G\textsubscript{\alpha}2.

To further test whether the disrupted interaction between 5-HT1BR and G\textsubscript{\alpha}2 observed with mutant 5-HT1BR is truly due to lacking modulation by GSK3\(\beta\), GSK3\(\alpha\), and GSK3\(\alpha\) were eliminated from cells by GSK3\(\beta\)- and GSK3\(\alpha\)-targeting shRNAs, respectively (Fig. 2A). Complete GSK3\(\beta\) knockdown abolished both serotonin-induced transient stabilization (Fig. 2B) and serotonin pretreatment-induced reduction (Fig. 2C) of 5-HT1BR-G\textsubscript{\alpha}2 interaction, and a partial GSK3\(\beta\) knockdown sufficiently blocked the transient stabilization and moderately diminished the serotonin pretreatment-induced reduction of interaction. Cells with GSK3\(\alpha\) knockdown or expression of a nontargeting shRNA, however, responded to serotonin similarly to cells expressing wild-type 5-HT1BR without shRNA (Fig. 2, B and C). Therefore, GSK3\(\beta\), but not GSK3\(\alpha\), modulates 5-HT1BR-G\textsubscript{\alpha}2 interaction. The effect of GSK3\(\beta\) was also confirmed in cells expressing the inactive p96A-GSK3\(\beta\) (Frame and Cohen, 2001) or K85,86A-GSK3\(\beta\) (Eldar-Finkelman et al., 1996), in which serotonin pretreatment did not reduce the BRET ratio between 5-HT1BR and G\textsubscript{\alpha}2, but cells expressing the constitutively active S9A-GSK3\(\beta\) (Sutherland et al., 1993) responded to serotonin similarly to cells with wild-type GSK3\(\beta\) (Fig. 2D). These data together suggest that serotonin-induced conformational change between 5-HT1BR and G\textsubscript{\alpha}2 requires the presence of active GSK3\(\beta\).

Activation of G\(_{\alpha}\)-protein links to both inhibition of cAMP (Raymond et al., 2001) and phosphorylation of Akt that represents activation of Akt (Hsu et al., 2001). We showed previously that inhibition of GSK3 abolishes 5-HT1BR-induced inhibition of cAMP production (Chen et al., 2009). Here, we further tested whether 5-HT1BR-induced phosphorylation of Akt also requires GSK3 interaction with 5-HT1BR. In wild-type 5-HT1BR-expressing cells, serotonin (1 \( \mu \text{M} \)) and the 5-HT1BR agonist anpirtoline (1 \( \mu \text{M} \)) significantly increased the levels of phospho-Thr308-Akt and phospho-Ser473-Akt without changing the level of total Akt (Fig. 3). The effect was blocked by the 5-HT1BR antagonist SB216641 but not by the 5-HT1AR antagonist WAY106655, and the 5-HT1AR agonist 8-OH-DPAT (1 \( \mu \text{M} \)) had a minimal effect on Akt in these 5-HT1BR-expressing cells. In contrast, serotonin and anpirtoline had a minimal effect on phospho-Thr308-Akt and phospho-Ser473-Akt in S154A mutant 5-HT1BR-expressing cells, suggesting that GSK3\(\beta\) interaction with 5-HT1BR is necessary for 5-HT1BR-induced phosphorylation of Akt.

Although coupling and activation of G\textsubscript{\alpha}2 is the canonical signaling of 5-HT1BR, many GPCRs have been shown to interact with the adaptor protein \( \beta\)-arrestin2 (Lefkowitz and Shenoy, 2005). In cells expressing 5-HT1BR-Rluc and \( \beta\)-arrestin2-YFP, the two proteins exhibited a low level of interaction at resting state (Fig. 4A). Serotonin pretreatment significantly increased BRET ratio in an YFP fluorescent intensity-dependent manner, reaching maximum at 0.5 \( \mu \text{g} \) of \( \beta\)-arrestin2 DNA. The serotonin-induced 5-HT1BR-\( \beta\)-arrestin2 interaction had a slow onset, reached maximal BRET ratio at 4 min of serotonin treatment, and lasted throughout the 10 min of real-time BRET recording (Fig. 4B). However, unlike the interaction with G\textsubscript{\alpha}2, both S154A and T158A mutant 5-HT1BR responded to serotonin similarly to wild-type 5-HT1BR, with a time-dependent increase in BRET ratio (Fig. 4C). Therefore, lacking GSK3\(\beta\) interaction with 5-HT1BR does not affect serotonin-induced recruitment of \( \beta\)-arrestin2 to 5-HT1BR.

Because GSK3 inhibitors have potential therapeutic implications (Cohen and Goedert, 2004; Li and Jope, 2010), the effect of GSK3 inhibitors on 5-HT1BR was further tested.
The ATP-competitive GSK3 inhibitor SB216763 (10 μM) itself had no effect on the resting state interaction between 5-HT1BR and G<sub>α<sub>2</sub></sub>β<sub>2</sub> but it completely abolished serotonin-induced transient stabilization of the interaction (Fig. 5A) and concentration-dependently abolished serotonin pretreatment-induced reduction in interaction (Fig. 5B). In contrast, SB216763 had no effect on serotonin-induced increase in 5-HT1BR and β-arrestin2 interaction (Fig. 5C).

**Fig. 2.** Effects of GSK3β-shRNA and inactive GSK3β on 5-HT1BR-G<sub>α<sub>2</sub></sub>β<sub>2</sub> interaction. A, HEK293 cells stably expressing 5-HT1BR-Rluc were infected with lentivirus-carried shRNA for GSK3β (b1, b2, b3), GSK3α (a1, a2, a3), or a nontargeting shRNA (CTL). Cell lysates from these shRNA-expressing cells and noninfected cells (CTL) were immunoblotted for total GSK3α and GSK3β. B, cells in A with an indicated shRNA were transfected with YFP-G<sub>α<sub>2</sub></sub>β<sub>2</sub>β<sub>2</sub>. BRET was measured in these cells after serotonin pretreatment (10 μM) for 15 min. Mean ± S.E.M., n = 3–6; *, p < 0.05 compared with no serotonin treatment (no 5-HT). C, BRET was measured in these cells after serotonin pretreatment (10 μM) for 15 min before BRET measurement. Mean ± S.E.M., n = 3; *, p < 0.05 in t test when serotonin-treatment in each type of shRNA-expressing cells were compared with no serotonin treatment (no 5-HT). D, 5-HT1BR-Rluc-expressing cells were cotransfected with YFP-G<sub>α<sub>2</sub></sub>β<sub>2</sub>β<sub>2</sub> and a wild-type or a mutant GSK3β (R96A, K85,86A, or S9A) and pretreated with serotonin (10 μM) for 15 min before BRET measurement. Mean ± S.E.M., n = 3; *, p < 0.05 in t test when serotonin treatment in each type of GSK3β-expressing cells were compared with no serotonin treatment (no 5-HT).

**Fig. 3.** 5-HT1BR-induced Akt phosphorylation. CHO cells stably expressing wild-type (1BR-WT) or S154A-mutant (1BR-S154A) 5-HT1BR were treated with the 5-HT1BR antagonist SB216641 or the 5-HT1AR antagonist WAY100635 (1 μM) for 30 min before serotonin (5-HT), anpirtoline, or 8-OH-DPAT (1 μM) treatment for 5 min. Cell lysates were immunoblotted for phospho-Thr308-Akt, phospho-Ser473-Akt, and total Akt. Data is expressed as average optical density of immunoblots. Mean ± S.E.M., n = 3 to 5; *, p < 0.05 in ANOVA compared with basal value (no drug treatment) in each experiment.
In 5-HT1AR-expressing cells, 5-HT1AR interacted with G\(_{\alpha_2}\) in a manner similar to that of 5-HT1BR with an oscillation that was transiently stabilized by serotonin (Fig. 5D), and the interaction was reduced with serotonin pretreatment (Fig. 5E). However, the GSK3 inhibitor SB216763 did not alter either of the serotonin-dependent change of interaction.

To identify additional GSK3 inhibitors that have high potency and selectivity in modulating 5-HT1BR signaling, we screened a group of benzofuranyl-3-yl-(indol-3-yl)maleimides that are capable of inhibiting GSK3 at various concentrations (Supplemental Table 1) (Kozikowski et al., 2007; Gaisina et al., 2009) and compared their effects with a known GSK3 inhibitor, AR-A014418 (Bhat et al., 2003). Similar to SB216763 (Chen et al., 2009) (Fig. 5), AR-A014418 concentration-dependently abolished 5-HT1BR and GSK3\(\beta\) interaction (Fig. 6A), serotonin pretreatment-induced interaction between 5-HT1BR and G\(_{\alpha_2}\) (Fig. 6B), and 5-HT1BR-mediated inhibition of cAMP production (Fig. 6C). Among the 14 benzofuranyl-3-yl-(indol-3-yl)maleimides, BIP-135 (Fig. 6A) and maleimide 6 (Supplemental Fig. 1A) had a potent effect in disrupting the interaction between 5-HT1BR and GSK3\(\beta\) either without or with serotonin treatment, and other maleimides had a moderate or biphasic effect (Supplemental Fig. 1A). All tested maleimides concentration-dependently reversed serotonin pretreatment-induced reduction in interaction between 5-HT1BR and G\(_{\alpha_2}\) but did not affect the interaction in the absence of serotonin (Fig. 6B; Supplemental Fig. 1B). However, among the four tested maleimides, only BIP-135 efficiently abolished the effect of serotonin in reducing cAMP production (Chen et al., 2009), reaching a maximal effect at 1 \(\mu\)M concentration (Fig. 6C). Maleimides 3 and 6 did not elicit a concentration-dependent effect, and maleimide 8 had a biphasic effect that did not agree with its effect on 5-HT1BR-GSK3\(\beta\) interaction (Supplemental Fig. 1A). Therefore, some but not all GSK3 inhibitors selectively modulate 5-HT1BR-regulated G\(_{\alpha}\)-cAMP signaling.

A major physiological function of active 5-HT1B autoreceptors in brain is to negatively regulate serotonin release (Göthert et al., 1987), which has been reported as a G\(_{\gamma}\)-dependent action (Ghavami et al., 1997). To test whether GSK3 inhibitors may affect this physiological function of 5-HT1BR, we measured 5-HT1BR-mediated inhibition of \(^{3}H\)serotonin release in mouse cerebral cortical slices. Activation of 5-HT1BR by anpirtoline (5 \(\mu\)M) caused a 60% reduction in potassium chloride (50 mM)-evoked serotonin release (Fig. 7, A and B). Pretreatment of cortical slices with the GSK3 inhibitor AR-A014418 (5 \(\mu\)M) or the maleimide...
GSK3 inhibitor BIP-135 (1 µM) completely abolished the inhibitory effect of anpirtoline on serotonin release (Fig. 7B). 5-HT1BRs participate in different serotonin-induced behaviors via both autoreceptors and heteroreceptors (Sari, 2004). Enhancing serotonin release by ablating 5-HT1B autoreceptor action reportedly facilitates 5-HT1BR agonist-induced reduction of immobility when tested in either the TST or forced swim test (FST) (O’Neill et al., 1996; Chenu et al., 2008). Because GSK3 inhibitors are able to maintain sufficient serotonin release by abolishing anpirtoline-induced activation of 5-HT1B autoreceptors, we tested whether GSK3 inhibitors may facilitate the effect of anpirtoline in the TST in mice. Systemic administration of anpirtoline (4 mg/kg i.p.) only mildly reduced the immobility in mice (Fig. 8A), which was better observed at the first 4 min of a 6-min test but did not separate from control mice at the last 4 min of the test. Infusion of the GSK3 inhibitor AR-A014418 (0.3 nmol/mouse i.c.v.) itself had no effect on the TST, but when in combination with anpirtoline, the two drugs caused a significant reduction in immobility compared with control mice, and the combined effect seemed to be stronger at the last 4 min of the test. BIP-135 (0.08 nmol/mouse i.c.v.) itself also did not have an effect in the TST, but when combined with anpirtoline, the two drugs elicited a strong and significant effect in reducing immobility compared with either control or anpirtoline alone, and the effect sustained throughout the last 4 min of the 6-min test.

In contrast to the TST, AR-A014418 and BIP-135 had no

![Figure 5](image_url)
effect in anpirtoline-induced increase in locomotor activity (Fig. 8B), which is mainly a 5-HT1B heteroreceptor-mediated behavior (Pranzatelli et al., 1987; Cheetham and Heal, 1993). Because GSK3 inhibitors had no effect on 5-HT1BR-β-arrestin2 interaction (Fig. 5C), we tested whether the GSK3 inhibitor-insensitive locomotor activity is a β-arrestin2-dependent behavior. In β-arrestin2 knockout mice (Bohn et al., 1999), anpirtoline did not cause a detectable increase in locomotor activity at the time of treatment, and the total distance traveled after anpirtoline treatment was not significantly different from saline treatment in β-arrestin2 knockout mice (Fig. 8C). Therefore, 5-HT1BR-mediated locomotor activity at least partly depends on the presence of β-arrestin2, a signaling mechanism that is independent of 5-HT1BR interaction with GSK3β.

**Discussion**

After our previous finding that GSK3 selectively interacts with 5-HT1BR (Chen et al., 2009), this study further dissected the functional aspects of this interaction on 5-HT1BR-regulated signaling, physiology, and behavior. The study results suggest that GSK3β selectively modulates Gαi2-associated 5-HT1BR signaling pathways but not recruitment of β-arrestin2 to active 5-HT1BR; GSK3β only affects the activation state of 5-HT1BR, with little effect on the resting state; GSK3 inhibitors modulate 5-HT1B autoreceptor-regulated serotonin release; and GSK3 inhibitors differentially modulate 5-HT1BR-mediated behaviors.

Because Gαi is a major mediator of 5-HT1BR-regulated signaling (Raymond et al., 2001), this study began by testing whether GSK3 affects 5-HT1BR-Gαi2 interaction. The cell-based BRET assay is used because it allows studying dynamic protein interaction of GPCRs with G proteins (Angers et al., 2000). BRET assay clearly detected three interactive states of 5-HT1BR with Gαi2, the oscillatory interaction at resting state, the transient stabilization of interaction immediately after receptor activation by serotonin, and a drastic conformational change shortly after removing serotonin from

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**Fig. 6.** Selection of GSK3 inhibitors for 5-HT1BR interaction with GSK3β and Gαi2 and regulation of cAMP. HEK293 cells expressing 5-HT1BR-Rluc and YFP-GSK3β (A) or YFP-Gαi2 (B) were pretreated with the GSK3 inhibitor AR-A014418 or a maleimide BIP-135 at indicated concentrations for 2 h. Some cells were pretreated with serotonin (5-HT, 10 μM) for 15 min, followed by measuring BRET. n = 3–6; *, p < 0.05 in ANOVA when each concentration of an inhibitor treatment was compared with no inhibitor treatment. C, CHO cells transfected with 5-HT1BR were pretreated with the indicated concentrations of AR-A014418 or a maleimide for 2 h, followed by serotonin (10 μM) for 30 min before stimulating cAMP production with forskolin (fsk; 10 μM, 15 min). The levels of cAMP (in picomoles) are normalized by the protein concentration (in milligrams). n = 3–4; *, p < 0.05 in ANOVA when each concentration of an inhibitor treatment was compared with no inhibitor treatment.
the receptor. The interaction pattern in the absence or the presence of serotonin also applies between 5-HT1AR and Gαo, suggesting that this is a common dynamic interaction pattern between type I serotonin receptors and Gαo. A small oscillatory interaction was observed by the real-time BRET during receptor resting state, which we confirm is not a baseline noise of the BRET assay because it was invariably detected in all experiments using different 5HT1BR and Gαo DNA constructs. The oscillatory interaction between wild-type 5HT1BR and Gαo can be invariably stabilized by serotonin treatment, whereas there is no oscillation between 5HT1BR and YFP or between 5HT1BR and β-arrestin2. Nevertheless, because the serotonin-induced change of oscillatory interaction is small and transient, we also used the fixed-time BRET to measure the 5-HT1BR-Gαo interaction after serotonin treatment. This assay detected a nearly 60% reduction of BRET ratio only after serotonin was washed out. Because coimmunoprecipitation assay that measures a steady-state protein association did not detect a dissociation of 5-HT1BR and Gi, the serotonin-induced change of interaction seen in BRET assay is probably a dynamic protein conformational change between 5-HT1BR and Gαo that represents Gi activation. However, to fully interpret the relationship of changes observed by real-time and fixed-time BRET, other protein structural studies are required that will not be further discussed here.

Because all GSK3 manipulations applied in the study resulted in change of agonist-induced 5-HT1BR-Gαo interaction, it is confirming that GSK3β is a modulator of 5-HT1BR-regulated Gi signaling, and it provides extended explanation of our previous finding that 5-HT1BR-induced inhibition of cAMP production depends on the presence of active GSK3β (Chen et al., 2009). The selectivity of GSK3β on 5-HT1BR-Gαo interaction is likely by targeting 5-HT1BR, not Gαo itself, because the similar 5-HT1AR-Gαo interaction was not altered by GSK3 inhibitors.

Although we conclude that GSK3β is required for activation of Gαo-coupled signaling by 5-HT1BR, it should be acknowledged that the significant changes of 5-HT1BR response to agonists were mostly observed when GSK3β was removed from 5-HT1BR by receptor mutation, GSK3β knockdown, inactive GSK3β, and GSK3 inhibitors, whereas overexpression of constitutively active S9A mutant GSK3β had no additional effect on 5-HT1BR activity. This observation may suggest that the intrinsic active GSK3β is an integrative component of the 5-HT1BR, which associates with the resting 5-HT1BR and is required for full activation of 5-HT1BR by serotonin. On the other hand, inhibition of GSK3β may have significant implications in modulating 5-HT1BR-regulated signaling.

Gαo-mediated cAMP production is not the only GSK3-dependent signaling pathway of 5-HT1BR because activation of Akt by serotonin was significantly diminished in S154A-mutant 5-HT1BR-expressing cells. The mechanisms of regulating Akt by 5-HT1BR are not fully understood, but it is believed that both α- and βγ-subunits of G-protein as well as β-arrestin are associated with GPCR-induced activation of Akt (DeWire et al., 2007; New et al., 2007; Yang et al., 2009). The finding that GSK3 influences 5-HT1BR-induced activation of Akt further supports the significant effect of GSK3 in 5-HT1BR-regulated signaling. Because Akt is one of the upstream GSK3-regulating protein kinases that phosphorylate GSK3 at the N-terminal serine to inactivate GSK3 activity (Cross et al., 1995), the GSK3-dependent activation of Akt by 5-HT1BR could function as a feedback regulation to prevent the prolonged effect of GSK3 on 5-HT1BR, but this postulation remains to be examined.

To our knowledge, 5-HT1BR interaction with β-arrestin2 has not been reported, but this interaction is expected because β-arrestin has been recognized as an adaptor and signal transducer of many GPCRs (Lefkowitz and Shenoy, 2005). In this study, recruitment of β-arrestin2 to 5-HT1BR

![Graph A](https://example.com/graph_a.png)

**Fig. 7.** The effect of GSK3 inhibitors on 5-HT1BR-mediated inhibition of serotonin release. Potassium-evoked [3H]serotonin release was measured in freshly isolated mouse brain cerebral cortical slices. A, serotonin release was stimulated by potassium chloride (K+, 50 mM) twice. Before the second potassium stimulation, slices were treated with anpirtoline (ANP, 5 μM) for 5 min (right). Data is expressed as the percentage of baseline [3H]serotonin release. B, brain slices were pretreated with the GSK3 inhibitor AR-A014418 (5 μM) or BIP-135 (1 μM) for 58 min before addition of anpirtoline. Data are expressed as the ratio of second potassium-evoked peak (P2) to the first potassium-evoked peak (P1) of serotonin release. Mean ± S.E.M., n = 3–5 in each treatment; * p < 0.05 in ANOVA when values were compared with anpirtoline alone.
Fig. 8. Effects of GSK3 inhibitors on 5-HT1BR-regulated behaviors. A, male C57BL/6 mice (8–12 weeks old) were pretreated with AR-A014418 (AR, 0.3 nmol), BIP-135 (BIP, 0.08 nmol), or vehicle (CTL) (intracerebroventricular administration, 90 min), followed by anpirtoline (ANP 4 mg/kg i.p.) or saline treatment for 30 min before they were subjected to the TST. Immobility time was recorded and expressed either as the last 4 min of the 6-min test (left) or by each 2-min block (right). Mean ± S.E.M., n = 6 to 11 in each treatment group, p values indicate statistically significant difference in ANOVA. B, mice were treated with GSK3 inhibitors for 90 min and accommodated in the open field for 15 min before giving anpirtoline (4 mg/kg i.p.). Locomotor activity was represented by horizontal travel distance. Travel distance during each 5-min block was recorded (left), and post-treatment travel distance (30 min) is calculated as the ratio of pretreatment travel distance (15 min) (right). C, homozygous β-arrestin2 knockout mice with C57BL/6 background and strain-matched wild-type mice were accommodated in the open field for 15 min before giving anpirtoline (4 mg/kg i.p.). Horizontal travel distance was recorded as in B. Mean ± S.E.M., n = 6 to 11 in each treatment group; *, p < 0.05 in ANOVA compared with control (saline treatment).
was examined as the measure of a Gi-independent signaling that allows to test the signal pathway-selective effect of GSK3β. In strong contrast to 5-HT1BR agonist-induced activation of Gαs-cAMP signaling, removing or inhibiting GSK3β did not affect β-arrestin2 recruitment to 5-HT1BR, which strongly suggest that removing GSK3β does not make an inert 5-HT1BR; instead, GSK3β probably has functional selective modulating effect on 5-HT1BR.

To select GSK3 inhibitors that are effective in 5-HT1BR-regulated Gαs-cAMP signaling, it is important to sequentially test the 5-HT1BR-GSK3β interaction, 5-HT1BR-Gαs interaction, and cAMP production, because among all the GSK3-inhibiting agents tested in this study, only a few specifically abolish 5-HT1BR-mediated inhibition of Gαs-cAMP signaling. This could be due to the effect of some GSK3 inhibitors on other signaling mechanisms that regulate cAMP. The purpose of selecting GSK3 inhibitors with a specific effect on cellular action of 5-HT1BR is to determine whether these inhibitors may have potential for modulating 5-HT1BR functions in the brain. Although SB216763 (Smith et al., 2001), AR-A014418 (Bhat et al., 2003), and BIP-135 (Gaisina et al., 2009) have excellent GSK3 selectivity in cellular assays, SB216763 was not used for serotonin release and behavior tests because the effective concentration of this compound is higher, which limits its use in brain tissues.

We measured the effect of GSK3 inhibitors on serotonin release in mouse brain cerebral cortical slices because this is a representative function of 5-HT1BR autoreceptors that distribute at the axon terminals of serotonin neurons reaching to the cerebral cortex and other brain regions (Riad et al., 2000). 5-HT1BR-regulated serotonin release is a pertussis toxin-dependent action mediated by the inwardly rectifying potassium channel (Innis et al., 1988; Ghavami et al., 1997; Trillat et al., 1997). Because activation of the pertussis toxin targets Gαs, we hypothesized that the inhibitory effect of 5-HT1BR on serotonin release is a GSK3-dependent function. The finding that two highly effective GSK3 inhibitors blocked the inhibitory effect of anpirtoline on serotonin release suggests that GSK3 inhibitors are potential enhancers of serotonin release by minimizing the effect of 5-HT1BR autoreceptors. This could potentially be an important modulatory mechanism to balance the counteractive effects of 5-HT1BR autoreceptors and heteroreceptors.

Because behavior studies have been used to delineate the diverse functions of 5-HT1BR in brain (Clark and Neumaier, 2001; Miczek et al., 2004; Sari, 2004), we tested two well-known 5-HT1BR-regulated behaviors, TST and locomotor activity, as another functional measure to configure the role of GSK3 in 5-HT1BR function and to provide evidence for the in vivo significance of this GSK3 action.

5-HT1BR can reduce immobility in TST and FST (O’Neill et al., 1996; Chenu et al., 2008). This behavioral effect per se is a function of 5-HT1BR heteroreceptors located at nonserotonin neurons (Chenu et al., 2008), but the effect usually requires a high dose of 5-HT1BR agonist when administered via systemic route, because simultaneous activation of 5-HT1BR autoreceptors could suppress serotonin release and dampen this heteroreceptor-mediated behavioral effect. In this study, the TST was applied because it is suitable for experiments involving intracerebroventricular drug administration with cannula placement. We used the dose of anpirtoline that by itself had no significant effect in the TST, assuming that this treatment is at the interface in which the effect of 5-HT1BR heteroreceptors is mostly masked by simultaneous autoreceptor activation. The results that anpirtoline, when combined with a GSK3 inhibitor, significantly reduced immobility highly suggests that GSK3 inhibitors selectively control 5-HT1BR autoreceptor action but spare the actions of 5-HT1BR heteroreceptors. It should be noted that several GSK3 inhibitors were reported to reduce immobility when tested in the TST or FST (Li and Jope, 2010), but the GSK3 target of the behavioral effect is unknown. Therefore, it is critical that we used intracerebroventricular administration of GSK3 inhibitors at low doses that alone had no effect on the TST because the aim of this study is to test the modulating effect of GSK3 inhibitors on 5-HT1BR function with minimal influence on the off-target or non-neuronal effects of GSK3.

The selectivity of GSK3 inhibitors on 5-HT1BR-regulated behaviors is further supported by their lack of action on anpirtoline-induced locomotor activity, which is primarily a function of 5-HT1BR heteroreceptors at nonserotonin neurons (Pranzatelli et al., 1987; Cheetham and Heal, 1993) and by the diminished response to anpirtoline-induced locomotor activity in mice lacking β-arrestin2 that is independent of GSK3. However, the result of this study does not rule out the possibility that the dampened locomotor activity in β-arrestin2 knockout mice is a result of lacking dopamine D2 receptor activity, because D2 receptor-regulated locomotor activity is also a β-arrestin2-dependent action (Beaulieu et al., 2005).

Therefore, findings reported in this study provide new evidence that GSK3 is a functional selective modulator in 5-HT1BR-regulated Gαs signaling, serotonin release, and behavior. Future studies may further elucidate the function of GSK3 in serotonin neurotransmission and potential therapeutic applications of GSK3 inhibitors in neurological illnesses that are associated with altered 5-HT1BR activity.

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Authorship Contributions
Participated in research design: L. Chen, Zhou, P. Chen, and Li.
Conducted experiments: L. Chen, Zhou, Yang, and Li.
Contributed new reagents or analytic tools: P. Chen and Gaisina.
Performed data analysis: L. Chen, Zhou, and Li.
Wrote or contributed to the writing of the manuscript: L. Chen, Zhou, P. Chen, and Li.
Other: Li acquired funding for the research.

References


Hsu EH, Lochan AC, and Cowen DS (2001) Activation of Akt1 by human 5-hydroxytryptamine (serotonin)1B receptors is sensitive to inhibitors of MEK. J Pharmac Exp Ther 298:825–832.


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Glycogen Synthase Kinase-3beta is a Physiological and Behavioral Modulator of Serotonin 1B Receptors

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Supplemental Materials

Supplemental Table 1
Supplemental Figure 1
Supplemental Table 1. GSK-3β Inhibition by Benzofuranyl-3-yl-(indol-3-yl)maleimides

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</tr>
<tr>
<td>6</td>
<td>7-CH₂OCH₃</td>
<td>H</td>
<td>CH₃</td>
<td>0.23 ± 0.04°</td>
</tr>
<tr>
<td>7</td>
<td>5-OCH₃</td>
<td>H</td>
<td>CH₃</td>
<td>125.0 ± 35.0°</td>
</tr>
<tr>
<td>8</td>
<td>5-F, 6-Cl</td>
<td>7-OCH₃</td>
<td>CH₃</td>
<td>260 ± 32°</td>
</tr>
<tr>
<td>9</td>
<td>5-OBn</td>
<td>H</td>
<td>(CH₂)₃OH</td>
<td>220.0 ± 30.0°</td>
</tr>
<tr>
<td>10</td>
<td>5-Br</td>
<td>6-CH₂OH</td>
<td>CH₃</td>
<td>0.51 ± 0.05°</td>
</tr>
<tr>
<td>11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6-F</td>
<td>H</td>
<td>CH₃</td>
<td>566</td>
</tr>
<tr>
<td>12</td>
<td>1H-benzo[g]</td>
<td>5,6-difluoro</td>
<td>CH₃</td>
<td>314 ± 19°</td>
</tr>
<tr>
<td>13&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5,6-difluoro</td>
<td>H</td>
<td>CH₃</td>
<td>438</td>
</tr>
</tbody>
</table>

<sup>a</sup> Structure of Benzofuranyl-3-yl-(indol-3-yl)maleimides

<sup>b</sup> Published in (Gaisina et al., 2009; Kozikowski et al., 2007).

<sup>c</sup> Synthesized according to (Gaisina et al., 2009).

References


Supplemental Figure 1
Supplemental Figure 1. Selection of GSK3 inhibitors for 5-HT1BR interaction with GSK3β and Giα2, and regulation of cAMP. HEK293 cells expressing 5-HT1BR-Rluc and YFP-GSK3β (A) or YFP-Giα2 (B) were pre-treated with the GSK3 inhibitor AR-A014418 or a maleimide (BIP-135, M1-M13) at indicated concentrations for 2 hr. Some cells were pre-treated with serotonin (5-HT, 10 µM) for 15 min, followed by measuring BRET. n=3-6, *p<0.05 in ANOVA when each concentration of an inhibitor treatment was compared with no inhibitor treatment.